Cloning and characterisation of the Schizosaccharomyces pombe rad8 gene, a member of the SNF2 helicase family

Article (Published Version)

5964–5971 Nucleic Acids Research, 1993, Vol. 21, No. 25

Cloning and characterisation of the *Schizosaccharomyces pombe rad8* gene, a member of the SNF2 helicase family

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Received August 23, 1993; Revised and Accepted November 22, 1993 EMBL accession no. X74615

ABSTRACT

The *Schizosaccharomyces pombe rad8* mutant is sensitive to both UV and gamma irradiation. We have cloned the *rad8* gene by complementation of the UV sensitivity of a *rad8.190* mutant strain. The gene comprises an open reading frame of 3.4 kb which does not contain any introns and is capable of encoding a 1133 amino acid protein of 129 kDa. Deletion of the gene indicates that it is not essential for cell viability. Recognisable motifs are present for a nuclear localisation signal, a RING finger and helicase domains. The predicted protein is a member of the SNF2 subfamily of proteins and shows particular homology to the *Saccharomyces cerevisiae* RAD5 protein. Double mutant analysis demonstrated that the *rad8* mutant is not epistatic to mutants in the excision repair pathway (*rad13*) or checkpoint pathway (*rad9*). Analysis of radiation sensitivity though the cell cycle indicates that, unlike most other *rad* mutants, *rad8* is most sensitive to irradiation during the G1/S period.

INTRODUCTION

DNA repair mechanisms have important roles in the maintainence of the integrity of DNA. The fundamental requirement for these repair mechanisms is illustrated by a number of human genetic disorders which have been associated with defects in the repair of DNA, such as Xeroderma pigmentosum and Cockayne's syndrome. Many of the genes involved in these processes have been cloned from humans and from model systems, such as the budding and fission yeasts. A number of striking homologies have been identified between genes from different species (1). The degree of conservation appears to be particularly good for genes which contain putative helicase domains, e.g. the human genes *ERCC2* and *ERCC3* have high levels of sequence similiarity to their yeast homologues, and this homology is most apparent in the conserved helicase domains (2).

The fission yeast, *Schizosaccharomyces pombe*, has proved to be an important model system for the study of basic processes in higher eukaryotes, most notably cell cycle control (3). Eukaryotic DNA repair mechanisms have been most extensively studied in the budding yeast *Saccharomyces cerevisiae* (4,5), but the recent developments in the study of DNA damage responses in *S.pombe* now provides an important complementary model system. Radiation sensitive mutants in *S.pombe* were originally assigned to approximately 23 complementation groups (6,7), but more recent evidence suggests that not all of these are distinct (8). The functions of the corresponding genes is unknown in most cases, but they have been shown to be required for a number of different mechanisms including excision repair, e.g. *rad13* (9), *rad15* (10,11), *rad16* (8), error-prone repair, e.g. *rhp6* (12), recombination repair, e.g. *rhp51* (13), and mitotic arrest following DNA damage, e.g. *rad1*, *rad3*, *rad9*, *rad17* (14,15). Analysis of the cloned genes indicates that many of them possess significant sequence identity to DNA repair genes in budding yeast and man, e.g. *rad15*, which is homologous to *S.cerevisiae RAD3* and human *ERCC2* (10).

The *rad8* mutant was initially reported to be sensitive to UV and gamma irradiation, but not to be further sensitised by caffeine treatment after irradiation and to have a particularly low UV-induced mutation rate (6). This phenotype suggests that the *rad8* gene may be involved in recombination and/or error-prone repair processes. In this paper we report the cloning and characterisation of the *rad8* gene, which is capable of encoding a putative protein of 129 kDa with homology, predominantly within the helicase domains, to the SNF2 family of proteins. This is the first reported *S.pombe* gene which is a member of the SNF2 helicase family (16).

MATERIALS AND METHODS

Plasmids, strains and growth conditions

The *S.pombe* plasmids, pUR19 and pWH5, and the genomic library used in this study have been described elsewhere (17,18). The *S.cerevisiae RAD5*-containing plasmid pBM118 (19), and *RAD54*-containing plasmid pYEP13-RAD54 (20) were generous gifts from R.Johnson and D.Schild, respectively. Plasmids were grown in *E.coli* strain DH5a, (*endA1*, *hsdR17*, (*rk*$^-$,*mk*$^-$), *supE44*, *thi-1*, *recA1*, *lacIqZ-M15* (*lacproAB*)). M13 derivatives were grown in DH5aF′ (as DH5a except that it contains an integrated F′). *S.pombe* strains used in this study are shown in

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Table 1. Procedures and media used for the routine growth and maintenance of *S. pombe* strains were as reported in our previous work (21).

Irradiation of *S.pombe* cells

UV iradiation for survival curves of log phase asynchronous cultures was carried out directly on freshly plated cells using a Stratagene 'Stratalinker'. Survival curves represent the data from a minimum of four plates obtained from at least two separate experiments.

Gamma-irradiation was carried out in liquid YES medium at a cell density of 10^4/ml in a Gammacell 1000 using a ^{137}Cs source with a dose rate of 12 Gy/min.

Radiation survival through the cell cycle

S. pombe cultures were synchronised using lactose gradients as described by Barbet and Carr (22) using a procedure modified from Mitchison and Carter (23). Cells from the top of the gradient, which are in G2, were innoculated into fresh YES medium. At 15 minute intervals samples were taken for the following treatments. UV irradiation: 10^3 cells were plated and irradiated under a UVC lamp with a dose rate of 6 J/m^{-2}. Gamma irradiation: 0.1 ml of cells at a density of 10^4 cells/ml in YES were irradiated in a Gammacell 1000 ^{137}Cs source (dose rate 12 Gy/min) prior to plating. Control: 5×10^2 cells were plated as a negative control for each time point; mitotic and septation indices: a sample of cells were fixed in 80% methanol, air dried onto a microscope slide, stained with DAPI (0.1 μg/ml) and Calcofluor (0.5 μg/ml) and observed by fluorescence microscopy.

Genetics and molecular biology methods

S.pombe genetic and molecular biology methods have been described in our previous work (21). General molecular biology protocols were as detailed in Sambrook *et al.* (24).

RESULTS

Isolation of the *rad8* gene

An *S.pombe* genomic library (17) was used to transform the *rad8* mutant strain, sp.559, to uracil prototrophy. Approximately 50,000 *ura*$^+$ colonies were pooled, plated at a density of 1×10^4 cells per plate on selective medium and subjected to UV irradiation at a dose of 100 J/m^{-2}. After two further rounds of selection, individual colonies were tested for co-instability of the *ura*$^+$ and *rad*$^+$ phenotypes. The correcting plasmid, pRAE2, was extracted from UV-resistant transformants and used to transform the *E.coli* strain DH5a. Retransformation of the *rad8* mutant strain sp.559 with pRAE2 restored the UV sensitivity to approximately wild-type levels.

pRAE2 was found to contain a 4.8 kb insert (Figure 1A), and to localise the extent of the *rad8* gene within this fragment, a variety of subclones were constructed and tested for their ability to complement the UV sensitivity of the *rad8* mutation. The following fragments were subcloned: *Pst*I−*Bam*HI 3.4 kb, *Bam*HI−*Bam*HI 1.4 kb, *Hin*dIII−*Hin*dIII 2.5 kb and *Eco*RI−*Eco*RI 2.1 kb (Figure 1B). None of these subclones was able to complement the *rad8* mutation. This functional analysis indicates that the *Bam*H1 site is located within an essential region of the *rad8* gene, and that the gene is not entirely contained within either the 2.1 kb *Eco*RI fragment or the 2.5 kb *Hin*dIII fragment.

Table 1. *S. pombe* strains.

sp.011	*ade6.704, leu1.32, ura4D18, h-*
sp.012	*ade6.704, leu1.32, ura4D18, h+*
sp.089	*rad21, h-*
sp.101	*ade6.704/ade6.704, leu1.32/leu1.32, ura4D18/ura4D18, h+/h+*
sp.118	*rad9::ura4, ade6.704, leu1.32, ura4D18, h-*
sp.138	*rad13::ura4, ade6.704, leu1.32, ura4D18, h-*
sp.145	*rad4, ade6.704, leu1.32, h-*
sp.151	*rad18, ade6.704, leu1.32, ura4D18, h-*
sp.559	*rad8.190, ade6.704, leu1.32, ura4D18, h+*
sp.574	*cdc25.22, ura4D18, h-*
sp.628	*cdc12, h-*
Strains Created For This Study:	
sp.152	*rad8.190, ade6.704, leu1.32, ura4D18, h-*
sp.183	*rad8/rad8::ura4, ade6.704/ade6.704, leu1.32/leu1.32, ura4D18/ura4D18, h+/h*
sp.187	*rad8::ura4, ade6.704, leu1.32, ura4D18, h+*
sp.189	*rad8::ura4, ade6.704, leu1.32, ura4D18, h-*
sp.199	*rad8::ura4, rad21, h-*
sp.201	*rad8::ura4, rad13::ura4, ade6.704, leu1.32, ura4D18, h+*
sp.203	*rad8::ura4, rad9::ura4, ade6.704, leu1.32, ura4D18, h+*
sp.204	*rad8::ura4, rad4, ade6.704, leu1.32, h+*
sp.206	*rad8::ura4, rad18, ade6.704, leu1.32, ura4D18, h-*

Chromosomal mapping of pRAE2 to the *rad8* locus

The *rad8* locus has been reported to be on the most telomere proximal *Not*I fragment on the left arm of chromosome 1 (25). To map the *rad8* locus more precisely, crosses were carried out between a *rad8* mutant strain, sp.559, and a *cdc12* mutant strain, sp.628, or a *cdc25* mutant strain, sp.574. Analysis of the UV- and temperature-sensitive phenotypes of the resulting progeny indicated that the *rad8* gene maps 8cM on the telomere proximal side of the *cdc12* gene. To confirm that the cloned gene is the *rad8* gene, rather than a suppressor of the *rad8* mutant phenotype, its chromosomal location was mapped. A stable integrant of the pRAE2 plasmid in the *rad8* mutant strain sp.559 was obtained and crossed to the *rad*$^+$ strain, sp.011. Analysis of the resultant spores showed that no UV sensitive isolates were obtained. This indicates that pRAE2 had integrated at the *rad8* locus and thus contains the *rad8* gene. The *rad8* gene has also been hybridised to the gridded *S.pombe* cosmid array of Lehrach and colleagues and its position on the physical map has been reported (26), further defining the *rad8* locus.

Gene deletion

In order to test whether the *rad8* protein is required for cell viability, a null allele was constructed. A plasmid construct was made in which the 0.8 kb *Bcl*I−*Bcl*I fragment of the *rad8* ORF (Figure 1A) was replaced with a 1.7 kb marker fragment containing the *ura4*$^+$ gene (27). A linear 5.4 kb *Hin*dIII fragment containing this disrupted region was then used to transform the diploid strain sp.101 to uracil prototrophy. Stable *ura*$^+$ colonies were isolated and Southern analysis was used to confirm that a single copy of the *ura4*$^+$gene had integrated at the *rad8* locus (data not shown). An h^{90} derivative (a revertant of h^+ which produces 90% of cells able to sporulate on low

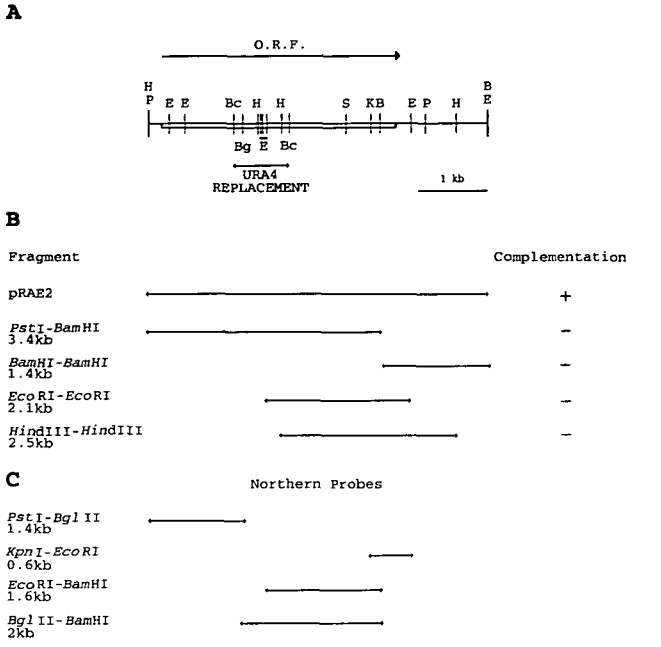


Figure 1. (**A**) Restriction map of the *rad8* region. The open box and arrow indicate the 1133 amino acid ORF. The thick line beneath 3 of the enzyme sites indicates 3 adjacent *Eco*RI sites. The restriction sites at the bold vertical line at both ends of the map represent flanking vector sites. The region replaced by a 1.7 kb fragment containing the *ura4* gene in the gene disruption experiment is indicated below the coding region. H=*Hin*dIII, E=*Eco*RI, Bc=*Bcl*I, Bg=*Bgl*II, S=*Sph*I, K=*Kpn*I, B=*Bam*HI, P=*Pst*I. (**B**) Diagram to show functional complementation data, drawn with respect to the restriction map above. Regions tested for ability to complement the UV sensitivity of the *rad8* mutant strain, sp.599, are represented by arrows. Ability to complement the defect is signified in the column on the right. (**C**) Diagram to show the fragments of pRAE2 used as probes in Northern analyses, drawn with respect to the restriction map above. Different fragments used are represented by arrows.

nitrogen agar plates) of the transformed strain was isolated and random spore analysis carried out. Haploid, *ura*$^+$, radiation-sensitive colonies could be isolated showing that the *rad8* protein is not essential for cell viability.

Radiation sensitivities of *rad8* strains

The survival of *rad8* strains was analysed after exposure to both UV and gamma radiation (Figure 2a and b). The *rad8* mutant strain sp.559 was found to be moderately sensitive to UV, but only slightly sensitive to gamma irradiation when compared to the wild-type strain sp.011. The radiation sensitivity of the *rad8* mutant was restored to approaching wild-type levels after transformation with the *rad8*-containing plasmid pRAE2. The survival, after irradiation, of the *rad8* deletion strain sp.187 was almost identical to the survival of the *rad8* strain sp.559.

DNA sequence analysis of the *rad8* gene

The DNA sequence of 3.9 kb of the pRAE2 insert was determined in both directions, using nested sets of deletions created by exonuclease III (28). The sequence has been submitted to the EMBL database and the accession number is X74615. Computer analysis of the sequence using the DNASTAR program revealed a single continuous open reading frame (ORF) of 3.4 kb which would encode a 1133 amino acid protein. The predicted protein has a M_r of 128,624 Da, a pI of 5.9 and contains 11% strongly basic and 13% strongly acidic amino acids and 32% hydrophobic and 32% polar residues. No potential intron splice sites were identified within this sequence. The first ATG of this ORF is 180 bp from one end of the pRAE2 insert.

The predicted *rad8* protein sequence contains several interesting features. There is a putative nuclear localisation signal at amino acid position 246 with the sequence **RKKSK** with two potential casein kinase II phosphorylation sites (29) in close proximity. One is at amino acid position 191 with the sequence **TPLD**, and the other is at amino acid position 286 with the sequence **TLME**. The protein sequence also contains the RING finger motif between residues 874 and 925 (Figure 3a). This sequence has been found in a number of other proteins, including the *S.cerevisiae* DNA repair proteins RAD18 and RAD5 (30,19). This type of zinc finger has been shown to bind both DNA and zinc *in vitro* (30).

Rad8 homologies

Database searches reveal that the *rad8* ORF has homology to the SNF2 helicase subfamily (16,31). This homology is largely confined to the helicase domains (Figure 3b). There are four DNA repair proteins reported to date as belonging to this group, namely the *S.cerevisiae* RAD54 (32), RAD16 (33,34) and RAD5 (REV2) (19,35) and human ERCC6 proteins (36). Comparison of the rad8 putative protein sequence with these proteins indicates that *S.cerevisiae* RAD5 more closely resembles rad8 than do the other proteins (Figure 3b and c). The predicted sizes of the rad8 and RAD5 ORFs are very close, 1133 and 1169 amino acids respectively. The two proteins have similar spacings of the helicase domains and both possess a RING finger motif (30) at approximately the same position (Figure 3c). Additionally, the phenotypes of both mutant strains are similar: *rad8* and *rad5* mutants are sensitive to both UV and gamma irradiation and are implicated in error-prone repair processes.

In order to test whether RAD5 can functionally substitute for rad8, a 4.4 kb fragment from the plasmid pBM118 (19) containing the *RAD5* gene, was subcloned into the *S.pombe* vector pWH5 (18) and used to transform the *rad8* deletion strain sp187. The *RAD54*-containing plasmid pYEP13-RAD54 (20) was also tested for the ability to complement the *rad8* deletion mutation. Transformants were selected for leucine prototrophy, replica plates were subjected to a UV dose of 300 J/m^{-2} and colonies grown up overnight. Examination of the irradiated plates showed that in both cases the transformants were more UV sensitive than wild-type cells. The survival of the transformants was tested at various doses of UV irradiation (Figure 2c) and confirmed that the *RAD54*-containing plasmid did not complement the *rad8* mutation. Transformants containing the *RAD5* plasmid showed an approximate four-fold increase in UV resistance at high doses when compared to the *rad8* deletion strain. This low level of complementation may be due to the fact that the RAD5 protein cannot functionally substitute for rad8 (a possibility which would seem likely since the level of homology between RAD5 and rad8 outside the helicase domains is very low) or that insufficient RAD5 protein is produced in the transformants.

Expression of the *rad8* gene

Northern analysis was carried out on total RNA (Figure 4) from wild-type (sp.011) cells. Using a 1.4 kb *Pst*I–*Bgl*II fragment from the 5′ end of the gene as a probe, four transcripts were detected in both preparations of RNA, with the sizes 4 kb, 3 kb, 2 kb and 1.1 kb (Figure 4, lane B). Similar results were observed with several RNA preparations probed with three other regions

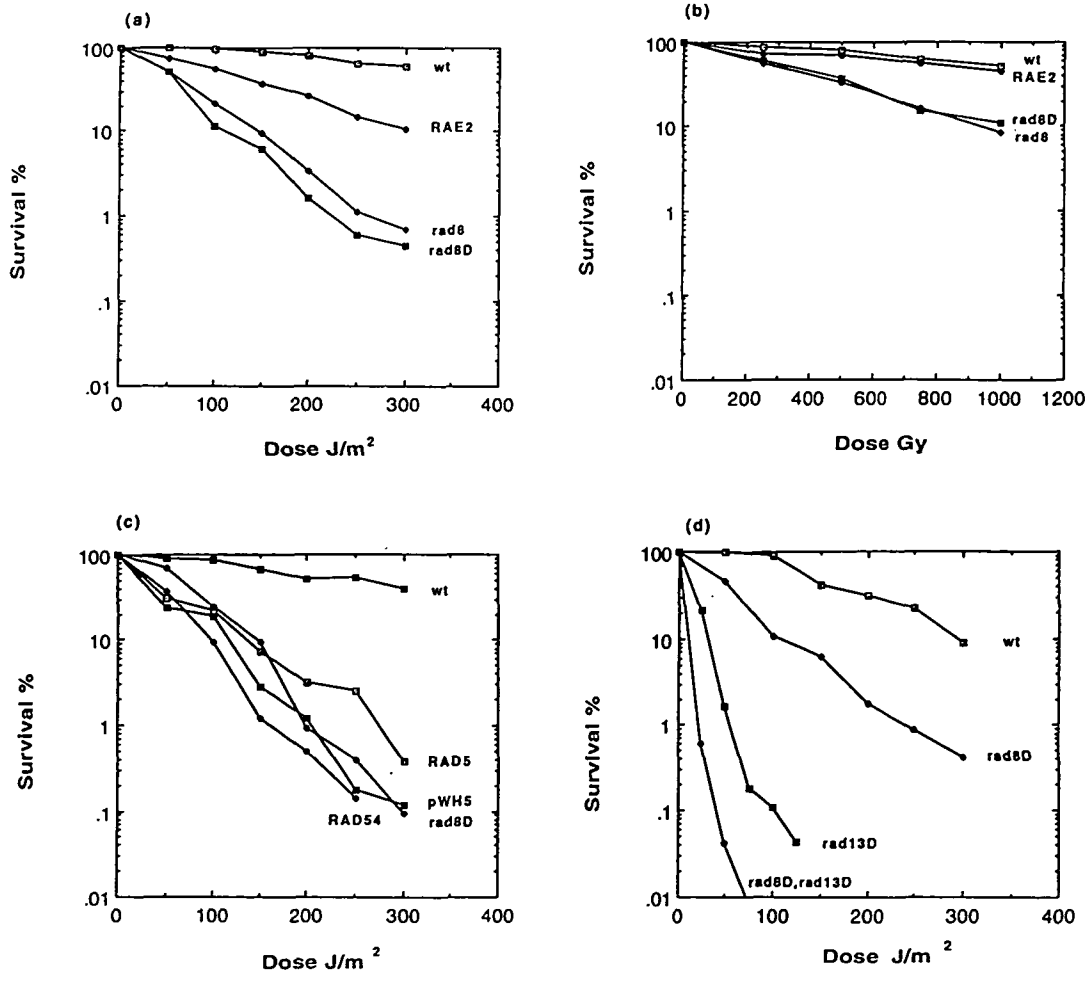
of the *rad8* gene (Figure 1C); namely an *Eco*RI−*Kpn*I 0.6 kb fragment from the 3′ end of the *rad8* gene, a *rad8* internal 2 kb *Bgl*II−*Bam*HI fragment and a *rad8* internal 1.6 kb *Eco*RI−*Bam*HI fragment, except that the smallest 1.1 kb RNA is not seen with either of the *rad8* internal fragment probes. Analysis of transcripts in the *rad8* deletion mutation strain (sp.187) indicates the absence of the 4 kb band (Figure 4, lane A), implying that this 4 kb RNA is transcribed from the *rad8* gene. This size is consistent with the size of the rad8 predicted ORF.

Radiation sensitivities of double *rad* mutant strains

Initial characterisation of the *S.pombe* DNA repair mutants classified them according to the UV and gamma sensitivities of the single mutants, since double mutant data required for epistasis analysis were limited (6). In order to determine the functional relationship of the rad8 protein with other rad proteins, a number of double mutant strains were isolated following the crossing of *rad8* mutants with the appropriate *rad* mutant strains and tetrad dissection. The UV survival of the double mutant strains was then assessed relative to each of the single mutants. A synergistic increase in UV sensitivity was observed when the *rad8* deletion mutation was combined with the *rad13* (sp.138) excision repair deletion mutation (9) (Figure 2d) or with the *rad9* (sp.118) checkpoint deletion mutation (14,21) (Figure 2e), as expected. Similarly, UV sensitivity was increased when the *rad8* deletion mutation was in combination with the *rad21* mutation (sp.089) (Figure 2f) which is defective in double-strand break repair (35), and also with the *rad18* mutation (sp.151) (Figure 2g) and the *rad4* (sp.145) (38) mutation (Figure 2h) which are involved in as yet undetermined processes. These observations suggest that the rad8 protein does not function in excision repair, and direct measurements on removal of cyclobutane and 6-4 pyrimidine photoproducts are in agreement with this (39). Similarly, the double mutant data are consistent with previous reports that rad8 does not seem to be involved with the G2 checkpoint pathway, although it has been noted that in a *cdc25* background, the survival of a *rad8* mutant after UV irradiation is increased if mitotic entry is delayed by incubation at the restrictive temperature for *cdc25* (14). Additionally, the rad8 protein does not function in combination with either the rad21, rad18 or rad4 proteins. Thus, on the basis of these epistatic data, we have not been able to define the pathway in which the rad8 protein acts, but the conserved excision repair and the checkpoint pathways have been excluded. We cannot rule out the possibility that the rad8 protein affects the efficiency of a process that still occurs in *rad8* mutants. However any such process must still operate in all the mutants tested.

S phase requirement for functional rad8 protein

To determine whether rad8 enhances DNA damage survival at a specific point in the cell cycle, experiments were carried out



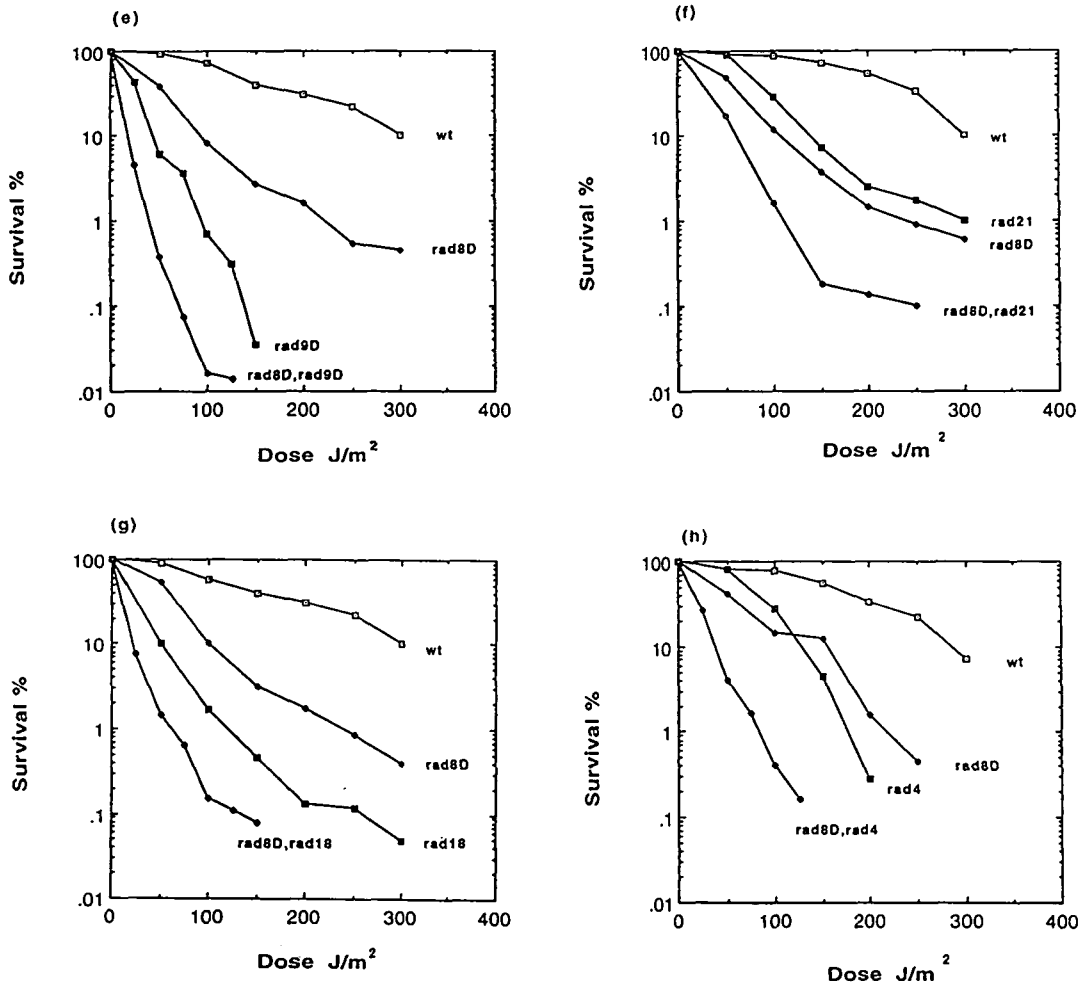


Figure 2. Survival curves of *rad8* strains following UV or gamma irradiation. (**a** and **b**) Survival of *rad*$^+$ strain sp.011 (wt), the *rad8* strain sp.559 (rad8), the *rad8* deletion strain sp.187 (rad8D) and *rad8* strain sp.559 containing the complementing plasmid pRAE2 (RAE2) after UV and gamma irradiation respectively. (**c**) Survival of *rad*$^+$ strain sp.011 (wt), the *rad8* deletion strain sp.187 (rad8), and the *rad8* deletion strain, sp.187 (rad8D), transformed with either pWH5 *S.pombe* cloning vector (pWH5), the *S.cerevisiae* RAD54 containing plasmid pYEP-RAD54 (RAD54) or the *S.cerevisiae* RAD5 gene cloned into pWH5 (RAD5), plated on selective minimal agar plates. (**d**–**h**) Survival of double mutants of the *rad8* deletion in combination with various other *rad* mutations. The *rad8* deletion (rad8D), *rad13* deletion mutant (excision repair) (rad13D), the *rad8*/*rad13* double deletion (rad8D,13D), the *rad9* deletion mutant (G2 arrest deficient) (rad9D), the *rad8*/*rad9* double deletion (rad8D,rad9D), the *rad21* mutant (double-strand break repair deficient) (rad21), the *rad8* deletion/*rad21* double mutant (rad8D,rad21), the *rad18* mutant (rad18), the *rad8* deletion/*rad18* double mutant (rad8D,rad18), the *rad4* mutant (rad4), the *rad8* deletion/*rad4* double mutant (rad8D,rad4).

to determine the UV sensitivity and gamma sensitivity of the *rad8* mutant through the cell cycle. Synchronous cultures of *rad8* deletion (sp.187), *rad13* deletion (sp.138) and wild-type (sp.011) strains were prepared using lactose gradients. At 15 min intervals samples of cells were removed, diluted, plated and UV irradiated. Simultaneously samples were taken to determine the septation index and the mitotic index. The results are shown in Figure 5. Several conclusions can be drawn from the results. Wild-type cells are most sensitive to irradiation during the G1/S period of the cell cycle and showed maximal resistance during G2. In contrast, the *rad13* deletion strain which has a defect in excision repair (9,39) shows maximal sensitivity to radiation at a time comensurate with mitosis. *rad8* deletion cells show a profile of sensitivity similar to that seem with wild type cells, being most sensitive to irradiation during the G1/S period of the cycle. *rad8* was the only mutant of ten tested (*rad2*, *rad4*, *rad8*, *rad9*, *rad13*, *rad17*, *rad18*, *rad21*, *rad26* and *rad27* (40)) which showed maximal sensitivity during G1/S phase, and this suggests that the rad8 protein may act to either repair or tolerate damage induced at this time. It should be noted, however, that the doses used in these experiments are relatively low (in order to limit the duration of their consequences to less than one cell cycle) and may not reflect the consequences of higher levels of damage administered at equivalent times.

DISCUSSION

We have cloned the *rad8* gene by complementation of the UV-sensitivity of a *rad8* mutant strain. The isolated plasmid, pRAE2, can restore both UV and gamma sensitivities to near wild-type levels and has been shown to map to the *rad8* locus, which is approximately 8cM proximal to the *cdc12* gene on chromosome 1. The transcript of the *rad8* gene is approximately 4 kb in size. Multiple transcripts are detected using *rad8* probes, as has been observed for several members of the SNF2 helicase gene family, for example, human *ERCC6* (41), *Drosophila Ldr* (42) and mouse

a.

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SPrad8   (874) ITECPICCNEPIQNPLLLN--CKHACCGDCLSEHIQYQKRRNIIPPLCHTCRQP (925)
SCRAD5   (911) SL..S..TT..MDLDKA.FTE.G.SF.EK..F.Y.EF.NSK.-LGLK.PN..NQ (963)
SCRAD16  (534) VVI.QL.ND.AEEPIESK---.H.KF.RL.IK.YVESFMEN.-NKLT.PV.HIG (583)
SCRAD18  (25)  LLR.H..KDFLKVPV.TP---.G.TF.SL.IRT.L-------.-NQ.N.PL.LFE  (68)
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b.

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DOMAIN I                                        DOMAIN Ia
rad8    524  GGILADEMGLGKTIEVLSL  542     573  LVVAPMSLL  581
RAD5    527  ....S.........VAAY..  545     589  .I.V.....  597
RAD16   205  ..V......M....QTIA.  223     234  .....TVA.  242
LDR     460  ......D......LTMI.S  478     528  ...C.A...  536
RAD54   330  .C.M.........LQCIA.  348     367  II.C.S..V  375
ERCC6   527  ....G.........QIIAF  545     570  VI.C.TTVM  578
SNF2    787  N.............QTI..  805     820  ..IV.L.T.  828

DOMAIN II
rad8    652  RVVLDEGHNIRNRESKTA  669
RAD5    678  .III.........TTV.S  695
RAD16   318  ..I...A...KD.Q.N..  335
LDR     599  .II...A.VV..HK.QSS  616
RAD54   451  LMLA....RLK.GD.L.F  468
ERCC6   642  Y.I.....K...PNAAVT  659
SNF2    890  EMII....RMK.AQ..LS  907

DOMAIN III
rad8    680  RWVITGTPIVNKLDDLYSLIKF  701
RAD5    706  K..L.....I.R.......V..  726
RAD16   346  ..CLS...LQ.RIGEM....R.  367
LDR     627  ..AL.....Q..EL.V.A.L..  648
RAD54   479  .VILS....Q.D.SEYFA.LS.  500
ERCC6   670  .IILS.S.MQ.N.RE.W..FD.  691
SNF2    919  .LIL....LQ.N.PE.WA.LN.  940

DOMAIN IV
rad8    741  VLRRTKETKD   750
RAD5    766  L.....QM..   775
RAD16   442  M.....VERA   451
LDR     683  M.....AQLQ   692
RAD54   551  II...NDILA   560
ERCC6   742  L...M.SDVK   751
SNF2    991  L...L.KDVE  1000

DOMAIN V
rad8    1029  DPDVNVLIISLKAGGVGLNLTCANHVFIMD  1058
RAD5    1067  YSRQKI.LL..............S.AYM..  1096
RAD16   686   NIQCE.FLV.......A...CE.SQ...L.   715
LDR     958   NNQKR..LL..T........IG...LLLL.  987
RAD54   722   EGQEFIFLL.S....C.I..IG..RLIL..  751
ERCC6   904   .TSIF.FLLTTRV..L.V...G..R.V.Y.  933
SNF2    1153  .SEYLCF.L.TR...L....QT.DT.I.F.  1182

DOMAIN VI
rad8    1068  QAIDRIHRLGQEKPVFVTR  1086
RAD5    1106  .....L..I..TNS.K.M.  1124
RAD16   725   .SG..V..I..YR..KI..   743
LDR     997   ..Q...Y.V..K.N.IIYK  1015
RAD54   761   ..LA.VW.D..K.DC.IY.   779
ERCC6   943   ..RE.AW.I..K.Q.T.Y.   961
SNF2    1192  ..Q..A..I..KNE.RIL.  1210
```

c.

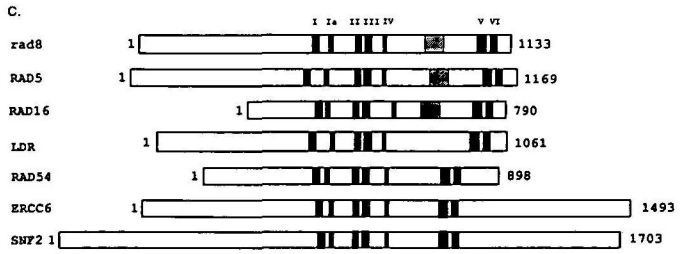

Figure 3. Homology of the rad8 putative protein to other proteins. (a) Alignment of putative RING Zn^{2+} finger binding regions in rad8 (SPrad8) and *S.cerevisiae* RAD5 (SCRAD5), RAD16 (SCRAD16), and RAD18 (SCRAD18). Residues conserved between a protein and the rad8 predicted protein are represented by a dot. The conserved cysteines and histidines are indicated with open boxes. Numbers in parentheses represent residue positions. (b) Alignment of the putative helicase domains of rad8 with other members of the SNF2 helicase subfamily, namely *S.cerevisiae* RAD5, RAD16, *Drosophila* Ldr, *S.cerevisiae* RAD54, human ERCC6 and *S.cerevisiae* SNF2. Residues conserved between a protein and rad8 are indicated by a dot. Numbers indicate residue positions. (c) Diagrammatic representation of rad8, RAD5, RAD16, Ldr, RAD54, ERCC6, and SNF2 putative proteins to show the relative positions of the homologous helicase and Zn^{2+} finger regions detailed in (a) and (b). Helicase domains are represented by black boxes, which are numbered at the top of the diagram and RING Zn^{2+} finger domains are represented by shaded boxes. Numbers at each end represent residue numbers.

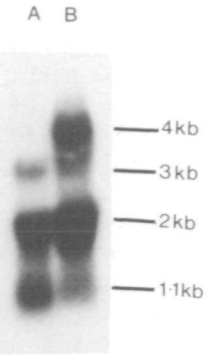

Figure 4. Northern analysis of *S.pombe* total RNA hybridised with a *rad8* specific, *PstI*−*BglII* 1.4 kb, probe. Lane A: total RNA isolated from a *rad8* deletion strain, sp.187; lane B: total RNA from a wild-type strain, sp.011. Four transcripts of 4 kb, 3 kb, 2 kb and 1.1 kb are seen in the wild-type strain. The 4 kb transcript is absent from the deletion strain; this is presumed to be due to premature termination of transcription in this strain.

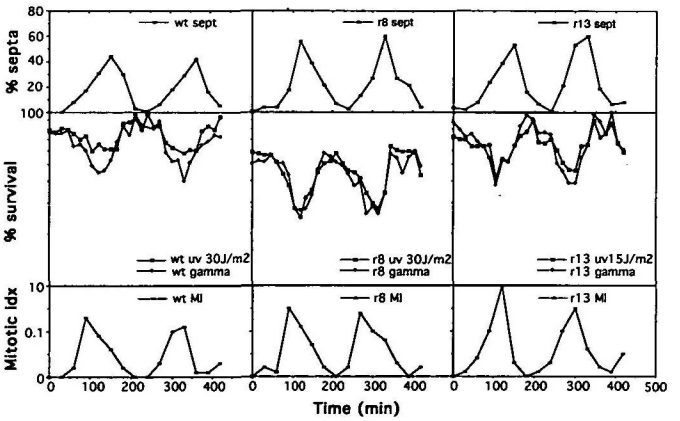

Figure 5. Cell cycle survival profiles. Cells were synchronised in G2 on lactose gradients and at 15 min intervals survival after UV and gamma irradiation was assayed. At 30 min intervals a sample was fixed and stained with DAPI and Calcofluor to determine the percentage of septated cells and the mitotic index. These provide an estimate of the position of cells in the cell cycle at the time of irradiation. The wild type and *rad8* deletion strain were irradiated with either 30 J/m^2 UV or 75 Gy ionising radiation. The *rad13* deletion strain was irradiated at 15 J/m^2 UV (to give equivalent percentage survival) or 75 Gy ionising radiation. Mitotic idx, mitotic index.

CHD1 (43), and *S.cerevisiae RAD54* (32). In most of these cases the phenomenon has been attributed to alternative polyadenylation. However, analysis of the transcripts in the *rad8* deletion strain implicates the 4 kb RNA as being transcribed from the *rad8* gene, since three of the transcripts are still observed in a *rad8* deletion mutation strain. The identity of the other three transcripts is not clear, but they may represent transcripts from other genes in *S.pombe* with homology to the *rad8* gene. We also find that the *rad8* transcript is induced at low levels after UV irradiation, (Shayeghi *et al.*, manuscript in preparation.) The related *S.cerevisiae* genes *RAD5* (*REV2*), *RAD16* and *RAD54* have also been reported as being UV-inducible.

Analysis of the *rad8* sequence predicts a protein of 1133 amino acids with a M_r of 129 kDa. This sequence contains several recognisable motifs. A putative nuclear localisation signal with associated casein kinase II phosphorylation sites suggests the rad8 protein acts in the nucleus, which is consistent with a DNA repair function. Phosphorylation at casein kinase II sites is thought to affect the rate of nuclear protein transport (29). A RING zinc finger motif has also been observed. This motif has been found in a number of proteins including some with roles in DNA repair and recombination (30). This suggests that the rad8 protein binds to DNA. The other major motifs identified in the sequence are seven helicase domains, including the ATPase motif, suggesting that the protein has DNA unwinding activity.

Homology searches reveal that rad8 is the first reported *S.pombe* member of a subfamily of proteins, found in a wide range of both prokaryotic and eukaryotic organisms, which show a particular type of conservation over the helicase regions (16,31). This subfamily is defined as proteins that have significant homology to the *S.cerevisiae* protein SNF2 (44), which was the first of the group to be identified and is the most well characterised. SNF2 is a transcriptional activator of numerous genes, possibly acting by altering chromatin structure, and is thought to form part of a large multi-subunit complex (45). ATPase, but not helicase, activity has been demonstrated for this protein (46). The family also contains other transcriptional activators, for example *Drosophila* Brm (47), and a negative transcription regulator, *S.cerevisiae* MOT1 (48).

Four DNA repair proteins have been reported with homology to SNF2, namely the *S.cerevisiae* proteins RAD54 (32), RAD16 (33,34), RAD5 (19), and human ERCC6 (36), which seem to represent four separate functions in repair of DNA. The human disease, Cockayne's syndrome, has been shown to be associated with a defective ERCC6 protein (35). This excision repair protein is thought to be associated with the preferential repair of lesions from the transcribed strand of active genes. *S.cerevisiae* RAD16 is also involved with damage excision but, in contrast to ERCC6, is thought to be required for the removal of damage from the inactive DNA. RAD54 is required for recombination repair and RAD5 for a postreplication repair pathway. None of these proteins is thought to be essential for cell viability. It has been speculated that the existence of this type of helicase in a number of different repair pathways may indicate a function required in each of the processes which must also have some pathway specificity. The RAD5 protein is the most similar to *S.pombe* rad8 (see Figure 2) but does not appear to complement efficiently the *rad8* deletion mutation. This may be because these two genes are not functional homologues. Alternatively, the two genes may encode proteins with similar functions, but which have diverged to an extent where they can no longer substitute for each other's function, e.g. in specific protein−protein interactions. The fact that homology between the two proteins is mainly confined to the helicase domains would support this. The increased radiation sensitivity of the *rad8* mutant during the G1/S period of the cell cycle is consistent with the previous report that the UV sensitivity of a *rad8* mutant can be partially rescued by transiently preventing cell cycle progression using the *cdc25.22* mutant following irradiation (14) and suggests that the mutant may be defective in a DNA damage response affecting DNA replication. However, since the null allele is viable, it is unlikely that the protein is required directly for replication. We are currently characterising the rad8 protein with a view to elucidating its precise role.

ACKNOWLEDGEMENTS

This work was supported in part by MRC grant G9102553CB (J.M.M. and F.Z.W.), and EC grant B17-0026 (A.M.C. and A.R.L). C.L.D. was supported by an SERC studentship no 90307820. J.M.M. and F.Z.W acknowledge the reciept of Royal Society and Wellcome Trust travel grants, respectively.

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